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Two distinct mechanisms alter p53 in breast cancer: Mutation and nuclear exclusion

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ABSTRACT Twenty-seven cases of inflammatory breast cancer were screened for the presence of the p53 protein by immunocytochemical methods using a monoclonal antibody directed against the p53 protein. Three groups were detected: 8 cases (30%) had high levels of p53 in the nucleus of the cancer cells; 9 cases (33%) had a complete lack of detectable staining; 10 cases (37%) showed a pattern of cytoplasmic staining with nuclear sparing. Nucleotide sequence analysis of p53 cDNAs derived from the samples with cytoplasmic staining revealed only wild-type p53 alleles in 6 out of 7 cases. An eighth case was determined to be wild type by a single-strand conformation polymorphism. In contrast, the samples containing nuclear p53 contained a variety of missense mutations and a nonsense mutation. The p53 cDNAs from 3 of the tumors that lacked detectable p53 staining were analyzed, and all 3 had wild-type nucleotide sequences. Interestingly, a case of normal lactating breast tissue also showed intense cytoplasmic staining for p53 with nuclear sparing. These data suggest that some breast cancers that contain the wild-type form of p53 protein may inactivate its tumor-suppressing activity by sequestering this protein in the cytoplasm, away from its site of action in the cell nucleus. The detection of cytoplasmic p53 in normal lactating breast tissue could suggest that this is the mechanism employed in specific physiological situations to permit transient cell proliferation. This observation could explain how some breast cancer tissues inactivate p53 function without mutation.

The wild-type p53 protein can act to negatively regulate cell proliferation (1-5) and can function as a suppressor of transformation (6, 7) and tumorigenesis (8). Cells that overexpress wild-type p53 are blocked near the G₁/S border of the cell cycle (3-5), suggesting a specific role for controlling cell replication. In many different human cancers, mutant forms of p53 proteins are present (9, 10) and these mutant p53 gene products no longer suppress cell division (11). In fact, p53 mutations are the single most common genetic change to be characterized in human cancers (9, 10).

Mutations at the p53 locus have been thought of as the most common mechanism to inactivate the negative regulatory effects of p53 upon cell proliferation. There are, however, several observations that do not comfortably fit with this idea. First, breast cancers contain p53 mutations in only about 30% of the cases (9, 10, 12-14), in contrast to colon cancers, 70% of which have p53 mutations (15, 16). Second, both colon and breast cancers show a reduction to homozygosity at the p53 locus in 70% of the cases (12, 15). While colon cancers retain the mutant p53 allele in 91% of the patients (15, 16), breast cancers retain the mutant allele in only 40% of the cases (12). Why 60% of such breast cancers select for a reduction to homozygosity at the p53 locus but retain a wild-type allele remains a mystery. Clearly, additional mechanisms are required to overcome or bypass the

wild-type p53 protein in these cancer cells. Several possibilities exist. First, there could be another tumor-suppressor gene that is genetically linked to the p53 locus and is inactivated by mutation and reduced to homozygosity. In this case, p53 would not play a major role in breast cancers. Alternatively, there could be another mechanism that inactivates the tumor-suppressor properties of the wild-type p53 protein.

The evidence presented in this communication supports the latter hypothesis. In some breast cancers the wild-type p53 protein is sequestered into the cytoplasmic compartment of the cell and stabilized. The exclusion of the p53 protein from the cell nucleus eliminates the ability of this protein to inhibit the proliferation of cells (17, 18) and therefore inactivates the p53 function independently of mutation. The mechanisms involved in this unusual cellular compartmentalization of p53 remain to be determined.

MATERIALS AND METHODS

Tissue. Patients were from French, Mediterranean, and North African backgrounds. Breast biopsy specimens were immediately quick-frozen in liquid N₂ and stored at -80°C. Tissue was also processed routinely and diagnosed as primary inflammatory breast carcinoma (IBC). Subsequently, all patients were treated with a combination of chemotherapy, radiotherapy, and hormonal manipulation (19). One specimen from Tunisia did not contain any carcinoma but showed normal lactating breast tissue. Normal tissue from reduction mammoplasties and reference tissues from noninflammatory invasive breast cancers were a generous gift from J. Marks, Duke University, North Carolina, and served as negative and positive controls for immunocytochemistry.

Immunocytochemistry. Adjacent frozen sections (4-6 µm) were cut, air-dried, and fixed in acetone for 10 min. p53 was detected with the anti-p53 monoclonal antibody PAb1801 (Ab-2, Oncogene Sciences, Manhasset, NY) at 500 ng/ml by following the procedure of Davidoff *et al.* (12). PAb1801 recognizes an epitope between amino acids 32 and 79 at the N terminus of both wild-type and mutant human p53 (20). With this protocol, PAb1801 showed a complete absence of nonspecific crossreactivity: both normal breast epithelium and the negative group of IBC cases did not reveal any stain. For negative controls, PAb1801 was replaced by normal mouse IgG at 500 ng/ml or by phosphate-buffered saline. Each case was assayed in three independent experiments. In addition, under identical conditions, selected tissues from all three groups were reacted with PAb240 (hybridoma supernatant), which recognizes a denaturation-sensitive epitope at amino acids 212-217, and CM-1 (1:1000), a polyclonal anti-serum against bacterially expressed human p53 (a gift from D.

Abbreviations: IBC, inflammatory breast carcinoma; SSCP, single-strand conformation polymorphism.

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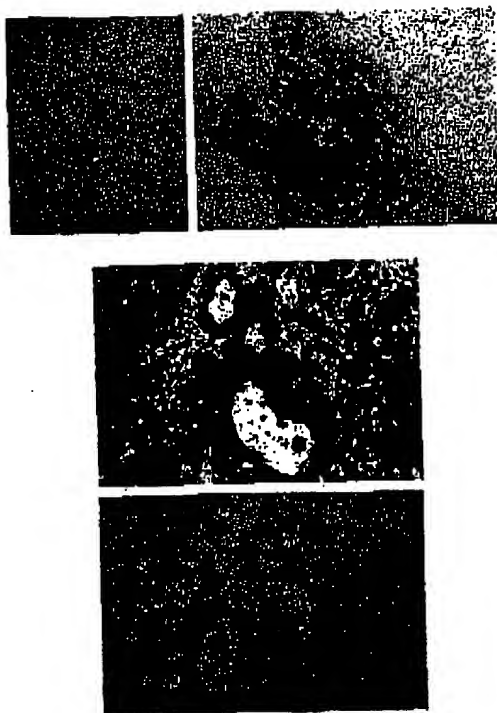


Fig. 1. p53 immunostaining exhibiting nuclear overexpression (a, c, and d, PAb1801; b, hematoxylin/eosin). b/c and d show two tumors (S113 and S199) carrying an identical codon 138 mutation. Note strong nuclear and diffuse cytoplasmic staining. b and c are adjacent sections of the same sample. Lymphocytes are unstained. ($\times 85$).

Lane) (21). The anti-cytokeratin monoclonal antibodies CY-90 (peptide 18) and K8.13 (peptides 1, 5–8, 10, 11, and 18) (Sigma) were also used. Adjacent frozen sections were stained with hematoxylin/eosin to evaluate the percentage of normal tissue contamination.

DNA Sequencing. Total RNA (2 μ g) served as a template for p53 cDNA synthesis with Moloney murine leukemia virus reverse transcriptase (BRL) (12). Five microliters of this reaction mixture served as cDNA template for PCR amplifications (22). For full-length sequencing, three overlapping

segments, consisting of codons 1–148 (exons 1–5, primers 5'-ATGGAGGAGCCGAGTCA-3' and 5'-ATCAACCCA-CAGCTGCAAGGG-3'), codons 118–353 (exons 4–10, 5'-GGGACAGCCAAGTCTGTGACT-3' and 5'-CCTGGGCA-TCCTTGAGTT-3' (12)), and codons 253–393 (exons 7–11, 5'-ACCATCATCACACTGGAAGACTCC-3' and 5'-ATGT-CAGTCTGAGTCAGG-3') were amplified. In the remaining cases, exons 4–10, the region to which virtually all missense mutations map, were sequenced (12). Sequences were confirmed on both strands and in some cases by analysis of single-strand conformation polymorphism (SSCP) (23). Two cases were analyzed solely by SSCP.

Hormone Receptors and c-erbB2 Analysis. Estrogen and progesterone receptor concentration was measured by the dextran/charcoal method (35). c-erbB2 amplification was determined by hybridization of a human c-erbB2 probe to Southern blots of tumor DNA (24).

RESULTS

Detection and Localization of p53 Protein in Breast Cancer Cells. To investigate the nature of the p53 gene and protein in a biologically homogeneous subtype of breast cancer, 27 specimens of primary IBC were obtained and frozen sections were prepared for immunocytochemical analysis using a p53-specific monoclonal antibody. Three distinct staining patterns were observed. (i) Eight specimens (30%) showed intense nuclear staining throughout the section or in foci of the cancerous tissue (Fig. 1a) as had been previously described (12). This result usually indicates the detection of high levels of mutant p53 protein in the nucleus. In 2 cases, a strong nuclear with weaker cytoplasmic localization was observed (cases S113 and S199) (Fig. 1b–d). (ii) Nine specimens (33%) showed complete absence of detectable p53 protein in multiple sections despite the presence of abundant malignant tissue (Fig. 2a and b). This result usually indicates that a very low level of wild-type p53 protein is present. (iii) In contrast, 10 cancer specimens (37%) showed a pattern of cytoplasmic p53 staining with sparing of the nuclei (Figs. 3 and 4). This pattern of p53 protein overexpression was widespread throughout the malignant epithelium and the staining intensity was moderate. Normal levels of p53 protein present in inflammatory and stromal cells (Fig. 1b and c and Fig. 2c and d) or in normal breast epithelium (Fig. 2c and d) were undetectable and thus served as internal control. Control experiments that substituted normal mouse IgG (Fig. 4d) or phosphate-buffered saline (Fig. 3d') for the p53-specific monoclonal antibody showed no detectable staining. These

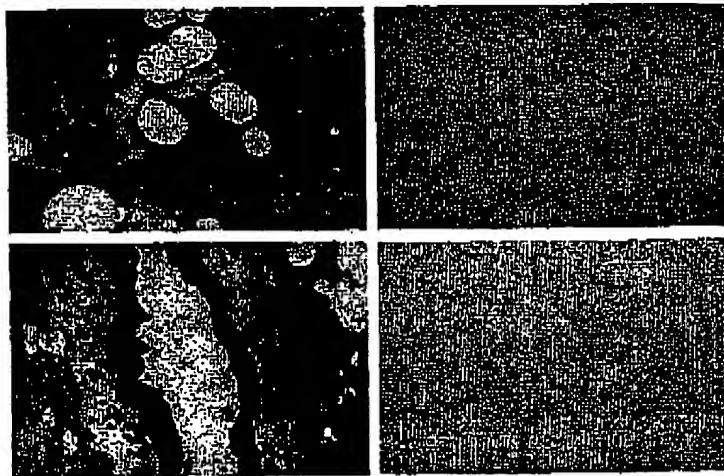


Fig. 2. (a and b) Tumor with no detectable p53 (b, PAb1801; a, adjacent hematoxylin/eosin-stained section). (c and d) Normal breast epithelium does not show p53 (d, PAb1801; c, hematoxylin/eosin). ($\times 90$).

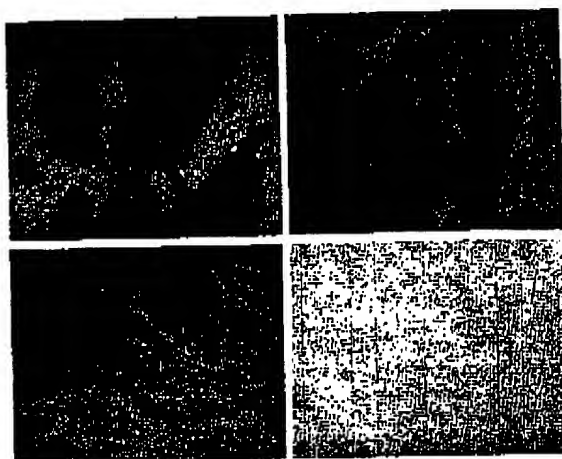


FIG. 3. Cytoplasmic overexpression of p53 in IBC case S219 (a, hematoxylin/eosin; b and c, PAb1801; d, phosphate-buffered saline control). (a, b, and d, $\times 85$; c, $\times 130$.)

experiments were carried out with a monoclonal antibody, PAb1801, that recognized both mutant and wild-type forms of the p53 protein. These data were confirmed by using a different monoclonal antibody, PAb240, directed against a different p53 epitope and a polyclonal antiserum (CM-1) directed against the human p53 protein. Although PAb240 is generally considered a p53 mutant-specific antibody, it has been shown that fixation or even freeze-thawing of tissue denatures the wild-type protein (or some percentage of the protein) to create a PAb240-reactive epitope (25). Thus, two distinct monoclonal antibodies and a polyclonal antiserum all gave the same patterns for p53 distribution. This excludes a nonspecific crossreactivity with cytoplasmic proteins such as cytokeratins. When the cytokeratin antibodies CY-90 and K8.13 were used to stain these tissue sections, they produced a distinctly different cellular staining pattern that was readily discriminated from the p53 pattern (Fig. 4e). The cytokeratin patterns of p53-positive and -negative tumors were identical.

The three staining patterns of p53 protein (nuclear, absent, cytoplasmic) have commonly been explained by the fact that mutant p53 proteins are more stable (have a longer half-life)

than wild-type p53 proteins (26, 27). The nondetectable levels of p53 in the p53-negative tumors are either the very low wild-type levels (see normal tissue in Figs. 1–5) or due to loss of p53 (deletion mutants). In contrast, the elevated levels of nuclear and cytoplasmic p53 proteins were previously thought to be mutant p53 proteins (25). However, direct nucleotide sequence comparisons between these different patterns of p53 protein distribution in tumor tissues have not been carried out previously on the same samples.

Correlation Between the p53 Expression-Localization Pattern and the Nucleotide Sequence of p53 cDNA. RNA was extracted from the tumor specimens, cDNA was made and amplified by PCR, and the product was directly sequenced. Five of six samples of breast cancers with high levels of nuclear p53 contained mutations (Table 1). Four of these samples contained p53 missense mutations in regions of the gene known to be targeted by other cancers (exons 5–8) (9, 10). One of the mutations (S234) resulted in a chain-termination codon at residue 342 producing a truncated stable protein in the nucleus. Two cases (S113 and S199) had an identical mutation at codon 138, where valine was substituted for alanine. Both of these cases showed diffuse nuclear and cytoplasmic staining (Fig. 1 b–d). Interestingly, the same mutation has been characterized in a mouse p53 protein (at codon 135). It produces a temperature-sensitive p53 protein with a mixed conformation of two-thirds mutant and one-third wild-type protein at 37.5°C which is accompanied by a dual localization in both the nucleus and cytoplasm in cell culture (4, 28). Another case (S248), for which no tissue sections were available, also bears a missense mutation, strongly suggesting nuclear accumulation of the protein (Table 1). Thus, the nuclear p53 protein observed at high levels results from a mutation in this gene. The single exception to this (S168) is a wild-type protein (sequenced at exons 4–10, residues 118–353) with a weak nuclear p53 staining reaction (Table 1). In this case, however, only 30% of the tissue was carcinoma and the poor representation of mutant p53 mRNA in the sample could have affected the ability to detect a mutant p53 sequence among the cDNAs.

Three cases with no detectable p53 protein revealed no mutations in the sequenced portion of the p53 message (exons 4–10). The cancerous tissue represented 70% or more of the sample, suggesting that the p53 alleles from the tumor tissue were wild type.

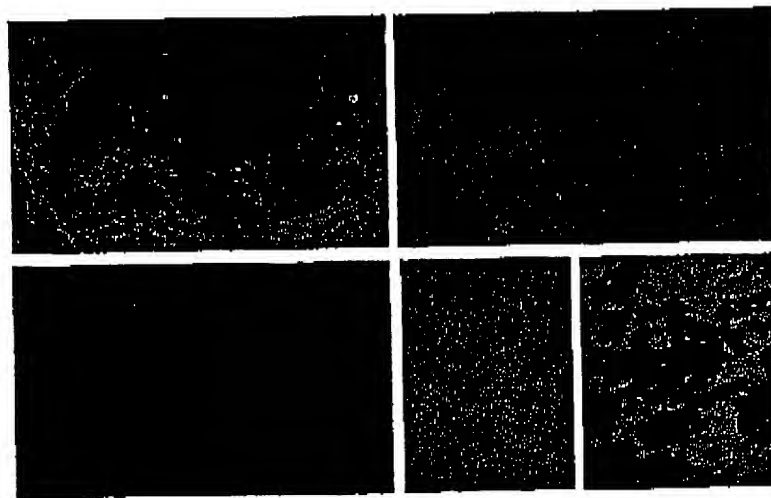


FIG. 4. Cytoplasmic overexpression of p53 in IBC case S225 [a, hematoxylin/eosin; b and c, PAb1801; d, mouse IgG control; e, anti-cytokeratin (CY-90) staining]. Note that the cytokeratin pattern differs from the p53 pattern. (a, b, and d, $\times 95$; c and e, $\times 150$.)

Table 1. Summary of cases of IBC with nuclear overexpression of p53

Case	% tumor	p53 staining	SSCP (exons 5-8)	Mutation (codons 118-353)	Amino acid change
S113	50	Nuclear, ++	Exon 5	138 GCC → G(C/T)C	Ala → Val*
S199	30	Nuclear, ++	ND	138 GCC → G(C/T)C	Ala → Val*
S150	100	Nuclear/focal, +	ND	249 AGG → AGT	Arg → Ser
S164	50	Nuclear, +	Exon 7	245 GGC → (G/T)GC	Gly → Cys
S168	30	Nuclear/weak, +	ND	None detected†	
S234	70	Nuclear/focal, +	ND	342 CGA → TGA	Arg → stop
S248	(No tissue available)		ND	285 GAG → AAG	Glu → Lys

Intensity of p53 staining is indicated (+, moderate; ++, strong). % tumor, percentage of tumor tissue in adjacent hematoxylin/eosin-stained section. ND, not done.

*Sequenced twice from independent PCR products; both cases showed strong nuclear staining with concomitant diffuse cellular staining.

†Seventy percent normal tissue contamination; *in situ* and invasive carcinoma.

Among seven cases of cytoplasmic overexpression of p53 (Table 2), six of the seven proteins were wild type in their nucleotide sequence. In each of these cases, the entire cDNA (residues 1-393) was sequenced. One case (S225) showed a silent, third-position polymorphism at codon 213 (CGA to CGG). In one case (T918), a three-base-pair, in-frame deletion at codon 241 (loss of a serine residue) was found to result in a cytoplasmic form of p53. An eighth case of cytoplasmic p53 (S253), which was not sequenced, was demonstrated to contain wild-type p53 by SSCP analysis (23) of exons 5-8. Almost all of these cases had only minimal stromal contamination and thus the p53 sequence analyzed represents the alleles analyzed from tumor tissue. Only one case (S225) had a significant admixture of lymphocytes. However, even a contamination of this range was well within the limits of sensitivity of the direct sequencing method to detect mutations. Surprisingly then, seven out of eight samples with p53 overexpression in the cytoplasm had a wild-type p53 sequence (Table 2). These data suggest that a mechanism other than mutation could be active to retain p53 protein in the cytoplasm of a cell and prevent it from negatively regulating cell proliferation.

Normal Lactating Breast Tissue. One of the specimens obtained contained only normal lactating breast tissue without any evidence of malignancy. Interestingly, it also exhibited a distinct cytoplasmic overexpression pattern for p53 in the milk-producing epithelial cells of the breast lobules (Fig. 5). [The sequence of the p53 cDNA (codons 118-353) was wild type.] This observation might suggest the existence of a normal physiological pathway that excludes p53 from the nucleus in breast epithelium that undergoes cell division followed by differentiation (lactation).

Clinical Correlations. All tumors were of the invasive ductal type, histograde 2 or 3. Seventy percent of the tumors with cytoplasmic overexpression of p53 were also estrogen receptor-positive, while only 20% of the nuclear overexpressors and 44% of the p53-negative group were estrogen

receptor-positive. The cytoplasmic (wild-type) p53 group had the longest overall survival, with an average of 38 months from the time of diagnosis (45 months if one patient who died immediately after diagnosis is not included), whereas the nuclear (mutant) p53 group had the shortest, with 20 months' survival. The p53-negative group ranked again in the middle, with a mean survival of 29 months. Although these data suggest that survival time and estrogen receptor status may correlate well with high levels of wild-type cytoplasmic p53 protein in these IBC patients, the group size is simply too small to draw firm conclusions at this time.

DISCUSSION

Breast tumors have shown two unusual patterns with regard to the presence of p53 mutations. First, only 30% of the breast cancers in women have p53 mutations (9, 10, 12-14), whereas 70% of colon cancers contain these mutations (9, 10, 15, 16). Second, in 91% of colon cancers that have only one p53 allele (reduced to homozygosity), only a mutant allele persists in the tumor (9, 10, 16). In contrast, 60% of breast cancers with one p53 allele retain the wild-type form of p53 (12). These observations and differences might be explained by the hypothesis that cancer cells of the breast can utilize a mechanism that is independent of mutation to inactivate the negative regulatory effects of the wild-type p53 upon cell proliferation (1-7) and tumorigenesis (8). The studies presented here support this hypothesis.

Of the 27 samples of breast cancer tissues analyzed by immunohistochemistry for subcellular location of p53, 30% exhibited high levels of nuclear p53 protein and most of these samples had mutant p53 proteins. The p53 protein normally acts in the nucleus and has several nuclear localization signals, which are necessary but not sufficient to direct it to this cellular location (17, 18, 29). The high levels of mutant p53 protein result from a longer half-life and greater stability of mutant p53 forms compared with wild type (26, 27). The

Table 2. Summary of cases of IBC with cytoplasmic overexpression of p53

Case	% tumor	p53 staining	SSCP (exons 5-8)	Sequence (codons 1-393)	Comments
S188	80	Cytoplasmic, +	ND	wt	
S219	80	Cytoplasmic, +	wt	wt	
S220	80	Cytoplasmic, +	wt	wt	
S225	50	Cytoplasmic, +	ND	wt	213 CGA → CG(A/G) (silent polymorphism)
S251	95	Cytoplasmic, +	ND	wt	
T902	70	Cytoplasmic, +	ND	wt	
T918	90	Cytoplasmic, +	ND	Codon 241	In-frame deletion of TCC (Ser)

+, Moderate; % tumor, percentage of tumor tissue in adjacent hematoxylin/eosin-stained section; wt, wild-type; ND, not done. An additional case, S253, contained wt alleles by SSCP.

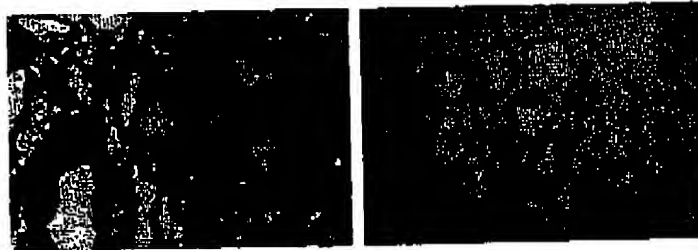


FIG. 5. Normal human lactating breast tissue (a, hematoxylin/eosin; b, PAb1801). ($\times 95$).

cancer specimens with no detectable p53 protein (33%) had only wild-type p53 nucleotide sequences (three samples tested) and did not stain with the p53 antibodies because of their low levels. Thirty-seven percent of the specimens examined had accumulated p53 in the cytoplasm and almost all of these cases revealed wild-type p53 sequences. One previous study on primary breast cancer detected cytoplasmic p53 protein, but the nucleotide sequence of mRNAs from these tumors was not determined (30).

The significance of detecting wild-type p53 protein in the cytoplasm of cancer cells becomes clearer when it is appreciated that p53 protein excluded from the cell nucleus no longer inhibits the proliferation of cells in culture (17, 18, 29). This protein must be in the cell nucleus to block cell division (5, 17, 18, 31). The observation (Fig. 5) that normal lactating breast tissue accumulates p53 in the cytoplasm of ductal cells suggests that estrogen-mediated cell division could involve inactivation of p53 protein via exclusion from the cell nucleus. In this case, the cancer cells might use an altered version of a physiological mechanism to retain p53 in the cytoplasm. While the mechanism by which the wild-type p53 could be retained in the cytoplasm is not known, an interesting hypothesis derives from the fact that one of the nuclear localization signals is located between amino acid residues 316 and 321 (17, 18, 29). Serine-315 can be phosphorylated by a cdc2-like kinase (32, 33). It is therefore possible that a growth-regulatory signal, such as an active cdc2-like kinase, could alter p53 protein via phosphorylation and exclude it from the nucleus. This is a testable hypothesis and further studies will be required to determine the mechanisms involved in the cytoplasmic retention of p53 in cancer cells. A similar level of regulation has been observed for the transcription factors NF- κ B, Rel and dorsal, whose access to the cell nucleus is controlled by nuclear uptake regulatory proteins (NURFs) (34).

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